

## Purification of Hypothalamic Corticotrophin-Releasing Factor (CRF) Of Ovine Origin. (30682)

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Hypothalamic control of the anterior pituitary by means of releasing factors appears to be well established and rapid advancements in this field have been made in the past few years. Although CRF was the first releasing factor to be described(1,2), its early history was marked by controversy, which stands in contrast to the other hypothalamic neurohormones where there has been general agreement between the results from various laboratories(3). We have already reported the purification and separate identity of luteinizing hormone-releasing factor (LH-RF) (4,5), follicle stimulating hormone-releasing factor (FSH-RF)(6), and growth hormone-releasing factor (GH-RF)(7). The present report deals with the preparation of highly purified hypothalamic CRF of ovine origin.

*Materials and methods. Extractions.* Acetone powders prepared from 2,000-4,000 ovine stalk-median eminences (SME's) were extracted with 4 M acetic acid, lyophilized, re-extracted with glacial acetic acid, diluted 3-4 times by volume with glass-distilled water and re-lyophilized. Details of this procedure have been described elsewhere(6). The lyophilized glacial acetic acid extract was used for further purification by gel filtration on Sephadex G-25.

*Gel filtration on Sephadex.* A preparative size column (150 × 4.5 cm) of Sephadex G-25 (fine grade) was employed for the first purification. The lyophilized, glacial acetic acid extract was dissolved in 0.1 M ammonium acetate buffer at pH 5.0. The clear supernatant was applied to the column which had been equilibrated with the 0.1 M ammonium acetate solvent. This ammonium

acetate buffer was used for elution and 20 ml fractions were collected. Conditions were similar to those used in our previous work (5-7). The zones with corticotrophin-releasing activity from several separate fractionations on the Sephadex column were combined, lyophilized, and redissolved in glacial acetic acid. After dilution with 3-4 volumes of glass-distilled water, the extract was re-lyophilized. This procedure was repeated twice and the final powder was stored in a vacuum desiccator over sodium hydroxide pellets and phosphoric acid in order to remove a large portion of ammonium acetate shown to interfere with retention of LH-RF on carboxy methyl cellulose (CMC)(5).

*Ion exchange chromatography.* Chromatography on a CMC column (2 × 100 cm) packed under 8 lb of pressure and equilibrated with 0.002 M (pH 4.5) ammonium acetate buffer was performed as described previously(5). The powder resulting from the gel filtration was dissolved in starting buffer and applied to the column. Elution was carried out by application of buffer of increasing pH and ionic strength through a 500 ml mixing flask. Gradient to pH 6.5, 0.04 M ammonium acetate buffer was applied at tube 10 and to 0.2 M buffer at tube 40. Twenty ml fractions were collected.

In the first run on CMC all of the CRF was not retained. Some activity appeared at the void volume. Consequently the contents of tubes with high peptide concentration appearing just after the void volume were re-lyophilized and rechromatographed.

Peptide concentration in the effluent from the columns was followed by the Folin-Lowry technique(8) and sodium and potassium concentration was measured by the Baird flame photometer.

*Bioassays.* Rats with median eminence lesions and diabetes insipidus were used for

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determining the corticotrophin-releasing activity in each fraction to be evaluated. An aliquot of 0.6-2.0 ml was injected intravenously (i.v.) into each of a group of 3-6 rats. One adrenal was removed prior to injection to provide a control value of adrenal ascorbic acid concentration. The second adrenal was dissected one hour after administration of extract in order to determine the adrenal ascorbic acid depletion. The occurrence of adrenal ascorbic acid depletion was taken as evidence that the extract contained either corticotrophin-releasing activity, or ACTH-like activity, or a mixture of the two activities. This procedure is that previously used in this laboratory (9).

The ACTH-like activity in the fractions was estimated by the adrenal ascorbic acid depletion which followed the iv administration of the fraction into hypophysectomized rats 48 hours post-hypophysectomy (10).||

Pressor activity was determined by Dekanski's method (11).

*Results. Gel filtration.* No tests for CRF activity were performed with the crude extract which was applied to the Sephadex column since previous studies had revealed that it contained ACTH and vasopressin (9) which would make it difficult to ascertain whether or not any adrenal ascorbic acid depletion was due to CRF. It is well known that vasopressin and ACTH can also deplete adrenal ascorbic acid (9,10).

Fractions from the Sephadex column which were capable of depleting adrenal ascorbic acid in rats with lesions were located in 2 zones. The first zone began near the void volume and extended over a wide number of tubes (Fig. 1). This area was wider than the zone found for other releasing factors (4-7). A second region which was active in depleting adrenal ascorbic acid was coincident with the peak of pressor activity, presumably caused by arginine vasopressin (Tube 112). The activity in this zone was similar to what could be expected from the quantity of vasopressin present. Consequently, no further studies have been performed with the fractions in this pressor zone. The adrenal ascor-

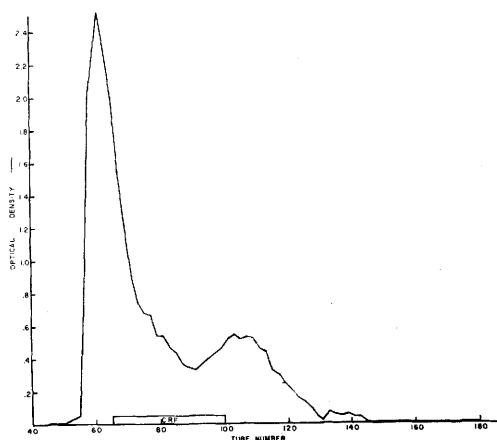


FIG. 1. CRF activity of ovine hypothalamic extracts obtained by gel filtration on Sephadex G-25. Optical density (at 750  $m\mu$ ) represents peptide concentration as determined by the Folin-Lowry reaction. CRF activity shown by the bar was estimated by adrenal ascorbic acid depletion in rats with hypothalamic lesions. Note that the activity was caused in part by ACTH-like substances since adrenal ascorbic acid depletion was also observed in hypophysectomized rats.

bic acid depleting activity in the early emerging zone described above (Tubes 65-106) was found reproducibly in all 4 fractionations; however, it was not caused exclusively by CRF since considerable ascorbic acid depletion was also observed in hypophysectomized rats in all tubes tested.

Rather than attempt to quantitate the proportion of the activity which could be attributed to CRF and to ACTH, respectively, the portion of this active zone which was eluted last from the column was selected for further chromatography on CMC. Since Sephadex separates substances primarily according to molecular size, this zone presumably contained the smaller molecular weight substances which should be more nearly free of ACTH or ACTH-like activity.

*Ion exchange chromatography on CMC.* After desalting by extractions with glacial acetic acid, the lyophilized active zones selected for further study from several fractionations on Sephadex were applied to the column. Two separate zones with adrenal ascorbic acid depleting activity in rats with lesions were eluted from the column in the first run on CMC (Fractionation #2-24) (Table I). The first active area was in tubes

|| Obtained from Hormone Assay Laboratory, Chicago, Ill.

TABLE I. ACTH-Releasing Activity of Fractions from CMC Columns.

Fractionation	Tube No.	Vol injected (ml)	Adrenal ascorbic acid depletion (mg/100 g adrenal)	
			Rats with ME* lesions	Hypophysectomized rats
2-24	13	1	124	( 2)
	40-49	2	2 ± 14	( 9)†
	50-59	2	0 ± 5	( 6)
	60-69	2	-1 ± 12	( 6)
	70	2	5	( 3)
	72-84	2	45.0 ± 8.7	(20)‡
	88-92	2	26 ± 12	( 5)
				5.6 ± 4.6 (14)
2-28	12-13	1	-18 ± 10	( 6)
	55-59	2	-4 ± 14	( 6)
	60-69	2	14 ± 11	( 6)
	70-80	2	-9 ± 13	( 6)
	83-89	2	59.6 ± 12	(10)§
	91	2	16	( 3)
	95-99	2	-2 ± 12	( 6)
ACTH	4mU	1		125 ± 7 ( 6)

\* Median eminence.

† Mean ± SEM (No. of test rats).

‡ P < .001 vs control tubes 40-70. P < .01 vs hypophysectomized rats.

§ P < .001 vs control tubes 12-80. P < .02 vs hypophysectomized rats.

which contained peptides that were not retained on the exchanger (*i.e.*, Tubes 13-15), whereas a second active zone was found in Tubes 72-84. These fractions were devoid of adrenal ascorbic acid depleting activity when administered to hypophysectomized rats at the same dose as that employed in rats with lesions.

The non-retained material from the first column was lyophilized, reappplied to the CMC Column, and chromatographed under identical conditions to those used before (Fractionation #2-28). This time no adrenal ascorbic acid depletion was elicited with fractions near the void volume (Fig. 2) (Table I). Several peaks of Folin-Lowry color were observed, but they were free of significant biological activity. Ascorbic acid depletion was observed in rats with lesions after injection of 2.0 ml aliquots of fluid from Tubes 83-89. Again, no detectable activity was found in hypophysectomized rats (Table I). These hypophysectomized rats were shown to be responsive to ACTH (Table I). Note that the Figure portrays the results from each tube evaluated, whereas the Table presents pooled results from two or more tubes in a given zone.

On the basis of the Folin-Lowry reaction, the peptide concentration of the dose of active

material injected was estimated to be < 3  $\mu$ g.

*Discussion.* The present results provide a method for preparation of highly purified CRF by preliminary extraction which is followed by gel filtration and ion-exchange chro-

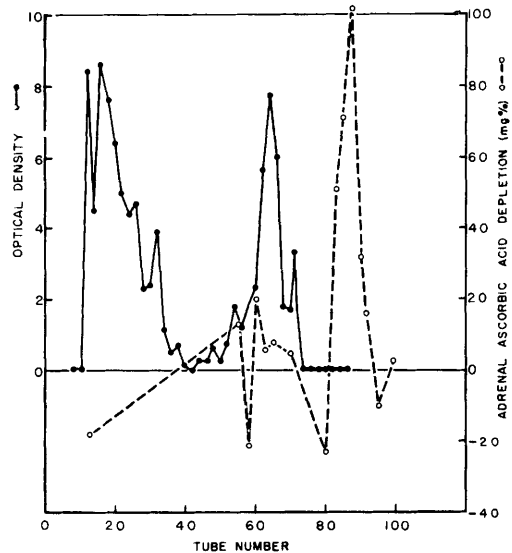


FIG. 2. CRF activity of fractions obtained from the CMC column (Fractionation 2-28). Gradients of ammonium acetate buffer of increasing pH and ionic strength were used for elution as indicated in the methods. Optical density represents peptide concentration. Adrenal ascorbic acid depletion in rats with hypothalamic lesions gives an estimate of CRF activity.

matography. The method should prove suitable for obtaining the large quantities of highly purified material needed for elucidation of the structure of hypothalamic CRF.

In the present work a highly specific, *in vivo*, bioassay for CRF has been used, the rat with an effective hypothalamic lesion. Previous studies have shown that such test rats respond only to vasopressin, CRF and ACTH, and fail to respond to a wide variety of non-specific stimuli, such as epinephrine, histamine, serotonin, substance P, and operative trauma(9,12). Furthermore, there has been a complete correlation in our laboratory between the response to CRF and vasopressin as measured by adrenal ascorbic acid depletion and the response as measured by rise in plasma corticosterone concentrations (DeVoe, Ridley, and McCann, unpublished). Since the active material obtained after chromatography on CMC was devoid of vasopressin, which had been removed by gel filtration, and of ACTH, as indicated by a complete lack of activity in hypophysectomized rats, we can be confident that it was indeed CRF.

CRF appears to be an entity distinct from the other hypothalamic factors which control anterior pituitary functions. The zone with adrenal ascorbic depleting activity obtained after gel filtration is free from FSH-releasing, LH-releasing and prolactin-inhibiting activity. It is contaminated by ACTH-like activity and by GH-releasing activity. A separation from these two activities was achieved by chromatography on CMC (Dhariwal, Kruulich, Antunes-Rodrigues, and McCann, in preparation).

Relatively few attempts to purify hypothalamic CRF have been previously reported (13-16). Royce and Sayers(13) reported a purification procedure which after preliminary extraction, employed chromatography on CMC with stepwise elution with ammonium acetate buffers of increasing ionic strength. They found, in agreement with our results on the first passage through CMC that CRF activity emerged in 2 zones, an early non-retained fraction and a later one which emerged when the 0.1 M buffer was applied. Our results with material already purified by gel filtration are in good agreement with

theirs. Since in our experience rechromatography led to complete retention of CRF on the exchanger, it is our belief that the failure of CRF to adhere to the exchanger completely in the first run was caused by the presence of cations such as Na and K in the extract. These have been shown to interfere with the adherence of the LH-RF to this cation exchanger(5). Although present in lesser concentration in the CRF zone than in the LH-RF zone, they may still have been sufficient to occupy a significant number of binding sites on the exchanger in the first experiment. These interfering cations presumably were reduced below the critical amount by the first passage through the CMC. Thus, the appearance of 2 zones with CRF activity in the first experiment with CMC should not be construed as evidence for the existence of 2 CRF's.

Sometime after the report by Royce and Sayers, Schally, Guillemin and their collaborators(14) also reported purification of hypothalamic CRF by means of chromatography on CMC. Recently, Schally and Guillemin (15) reported preliminary findings on hypothalamic CRF purified by gel filtration followed by counter-current distribution. Their results with gel filtration are in agreement with ours for they also found their active zone to be contaminated with ACTH-like activity after the Sephadex step. Their CRF separated into 2 zones, designated  $\alpha$  and  $\beta$  CRF after counter-current distribution. We, on the other hand, were able to locate only a single zone containing CRF after ion exchange chromatography with CMC which was completely devoid of ACTH-like activity.

The ACTH-like activity found after gel filtration may be caused by ACTH itself which contaminated the hypothalamic fragments (9, 13-15), and was eluted near the void volume of the column. In addition  $\alpha$  MSH has been shown to have corticotrophic activity and presumably contaminated much of the active zone as indicated by our tests and as shown previously by Guillemin and Schally(15).

The relationship, if any, between the hypothalamic CRF purified here and the various CRF's obtained from whole pituitary and posterior pituitary extracts by various work-

ers(2,3) remains to be determined. As pointed out earlier by several authors(2,3), posterior lobe powders probably constitute a poor starting material for concentration of CRF since they are maximally contaminated with ACTH, and vasopressin. Both of these substances interfere with CRF assays and vasopressin has intrinsic CRF activity(3).

*Summary.* Acetone powders were prepared from large numbers of ovine SME fragments. After preliminary extraction of the powder with glacial acetic acid and lyophilization, the resultant powder was extracted with 0.1 M ammonium acetate and subjected to gel filtration on a long column of Sephadex G-25. A wide zone was eluted from the column which induced adrenal ascorbic acid depletion upon its iv administration into rats with median eminence lesions. Part of this activity was caused by ACTH-like substances, since it was still present in hypophysectomized rats; however, this active zone was free of pressor activity. The active zones from several fractionations with Sephadex were pooled and placed on a CMC column. Elution was accomplished by application of gradients of ammonium acetate buffer of increasing ionic strength and pH. A narrow zone was eluted from the column which was active in inducing adrenal ascorbic acid depletion in rats with lesions, but was inactive in hypophysectomized rats. A dose of  $< 3 \mu\text{g}$  of peptide had significant activity. This method appears applicable for preparation of highly purified hypothalamic CRF. In this study we were

able to locate only one CRF; however, the possible existence of other CRF's is not excluded.

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### Certain Aspects of Protein Metabolism in Hypercholesteremic Chickens\* (30683)

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Persistent hypercholesteremia is likely to produce changes in the metabolic pathways of proteins. It was, therefore, our interest to study the free amino acids of serum and aorta and the glutamic-oxalacetic-transaminase (GOT) and glutamic-pyruvic-transaminase (GPT) in the serum, aorta and liver of

hypercholesteremic chickens. Liver was selected since it is an important site of trans-

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